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RESEARCH ARTICLE

Biological evaluation of 2,5-dimethyl-1,4-benzoquinone biotransformation metabolite

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Abstract

The substrate of this study, 2,5-dimethyl-1,4-benzoquinone (DMBQ), is one of the naturally occurring quinones which is found in insects as a defence metabolite. In the present study, DMBQ was biotransformed by using the micro-fungus *Corynespora cassicola*. The transformation metabolite was initially screened by TLC and GC-MS, then further characterized by NMR (^{13}C- and ^{1}H-NMR) spectroscopic techniques and was identified as '2,5-dimethyl 1,4-benzenediol'. In addition, both the substrate and metabolite was evaluated for the *in vitro* anticandidal, and antioxidant properties along with XTT-cytotoxicity.

Keywords: Benzoquinone, microbial biotransformation, biological activity, DPPH antioxidant activity

Introduction

Quinones are a large class of compounds endowed with rich and diverse chemistry. They are widely distributed in the natural world; present in bacteria, plants and arthropods and hence quinones are ubiquitous to living systems. Where these molecules are involved in vital cellular processes such as respiration and photosynthesis. A large number of chemical derivatives with 1,4-benzoquinone as the basic subunit exhibit prominent pharmacological applications such as antimicrobial, antitumor, antimalarial, antineoplastic, anticoagulant and herbicidal activity among others. Especially naturally occurring quinones have been reported with a range of activity against insect, fungal, weed and other agricultural pests (Mozaina et al., 2008). Quinones are widespread in the defensive secretion constituents of arthropods and the methylated quinones found in opilionids are of more limited occurrence (Eisner et al., 1977). Methyl- and ethylquinones are well established components of the defensive secretions in many arthropods and their presence vary between species and even sexes (Gunbilig & Boland, 2009). Many applications of quinones can also be found in the field of synthetic organic chemistry (Abraham et al., 2011; Gulaboski et al., 2013).

In the present study, we aimed to obtain new natural derivatives. Therefore, we used 2,5-dimethyl-1,4-benzoquinone as a substrate in the microbial biotransformation experimental. In addition, we also evaluated the in vitro anticandidal and antioxidant potential for both substrate and metabolite to the best of our knowledge for the first time.

Materials and Methods

General

2,5-dimethyl-1,4-benzoquinone was purchased from Sigma. Amphotericin-B (Sigma-Aldrich), chloramphenicol (Sigma) and Ketoconazol (Sigma-Aldrich) were used. All remaining chemicals were high purity and quality.

Microbial transformation

Biotransformation was performed using *Corynespora cassicola* (DSM 62474) in sterilized 100 mL Erlenmeyer flasks, each containing 25 mL of liquid medium (glucose, peptone, yeast extract, NaCl, and Na_2HPO_4; pH 7.0). Microorganism and substrate control flask sets were also included. Under aseptic conditions, 10 mg 2,5-dimethyl-1,4-benzoquinone was dissolved in sterile DMSO (Carlo-Erba) and was added into the culture suspension. All the flasks were incubated at 28 ºC using an orbital shaker (New Brunswick Scientific, USA) at 200 rpm for 1-14 days. Every 24 h, under aseptic conditions a 2 mL-sample of the medium was extracted three times with ethyl acetate. For the separation of the solvent, the samples were centrifuged at 4000 rpm for 3 min. After evaporation of solvent, samples were subjected to TLC (SiO_2; GF_{254}) and GC-MS systems for screening of the metabolites**.**

Purification by column chromatography

Preparative-scale biotransformations were carried out for the isolation and identification of metabolites. After 48 h pre-incubation, 1.0 g substrate in DMSO was added into the medium. After the incubation period, medium and mycelia were separated by a vacuum-filtration system (Sartorius), and both were extracted three times with ethyl acetate. The solvent was evaporated, the residue rich in metabolites was applied to silical gel column chromatography with ethyl acetate. The extract was eluted with hexane and ethyl acetate mixture (6:4, 4:6, 2:8, v/v), relevant fractions were combined according to TLC and GC/MS data.

GC-MS analysis

The GC-MS analyses were carried out with a Shimadzu QP2010 Plus system. Teknorama TRB-5MS (30m x 0.25 mm, 0.10 µm) was used with helium as carrier gas (1 mL/min). GC column temperature was kept at 60 °C for 5 min and then it reached to 260 °C at 20 min. Split ratio was adjusted at 50:1. The injector temperature was set at 250ºC. Mass spectra were recorded at 70 eV. Mass range was from *m/z 3*5 to 400.

NMR analyses

^{1}H NMR: Bruker DPX FT NMR (500 MHz), and ^{13}C NMR: Bruker DPX FT NMR (125 MHz) (solvent DMSO-d_6 Sigma-Aldrich), were carried out using both the substrate and metabolite.

Biological activity

The DPPH radical scavenging activity was performed according to Duymuş et al. (2014). Serial dilutions were carried out with the stock solutions (10 mg/mL) of the samples to obtain the concentrations of 10, 5, 2.5 x 10^{-1}, 1.25 x 10^{-2}, 62.5 x 10^{-3}, 31.25 x 10^{-4} mg/mL. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, gallic acid. The percentage inhibition was calculated using equation:

$$\text{Percentage Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] x100$$

Anticandidal activity

Anticandidal effects of the samples were evaluated by using partly modified CLSI (formerly NCCLS) micro dilution broth methods M27-A2, respectively. *Candida albicans* (clinical strain, Osmangazi University, Faculty of Medicine, Department of Microbiology and ATCC 24433), *C. glabrata* (ATCC 2001 & ATCC 66032), *C. tropicalis* (clinical strain and ATCC 1369) were used as the test microorganisms. Fluconazole (Sigma-Aldrich) were used as standard agents.

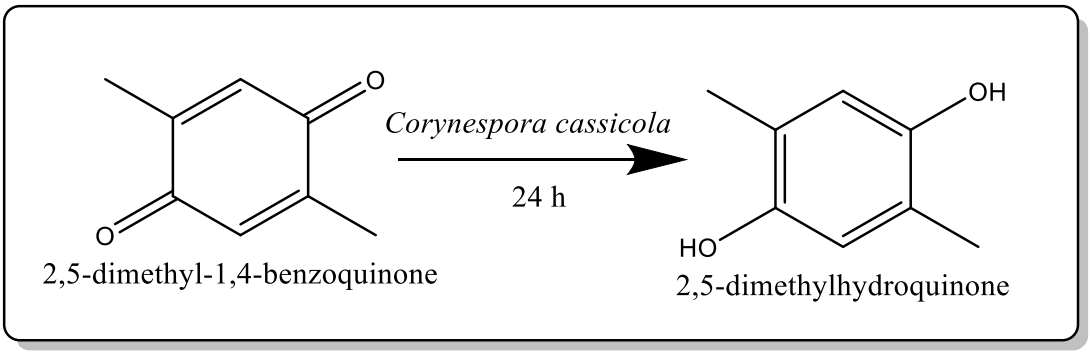
Cytotoxicity test

XTT assay, WSI human skin fibroblast (ATCC® CRL-1502™) cell line was used to investigate the cytotoxicity of the compounds. Cells were incubated according to the instructions of the supplier at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. WSI cells were seeded at $1x10^4$ cells into each well of 96-well plates. After 24 hours of incubation, compounds were added to the wells at the concentration range between 3.9-500 µg/mL concentrations in quadruplicates. The XTT assay (Xenometrix, Switzerland) was performed according to the manufacturer's instructions, after 24 h incubation with the compounds. The absorbance was determined after 2 h incubation at 480 nm with a reference wavelength of 680 nm using a microplate reader (BioTek, USA). Inhibition % was calculated for each concentration of the compounds according to the instructions of the manufacturer and IC_{50} values were estimated by plotting a dose response curve of the inhibition % versus test compound concentrations. The stock solutions of the compounds were prepared in DMSO and further dilutions were made with fresh culture medium. The final DMSO concentration was under 0.1%. All data were obtained from 3 independent experiments in quadruplicates.

Results and Discussion

The biotransformation metabolite was identified as 2,5-dimethylhydroquinone (syn: 2,5-dimethyl-1,4-benzenediol; 2,5-dimethylbenzene-1,4-diol), as shown in **Scheme 1**. Where the spectroscopic data was recorded as ^{1}H-NMR (500 MHz, DMSO-d^6): 2.00 (s, 6 H); 6.47 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ (ppm): 16.3 (C-7,8), 118.03 (C-3,6), 122.34 (C-5,2), 148,82 (C-1,4). And the molecular formula was $C_8H_{12}O_2$; confirmed by EI-MS: 138 (M+), 123, 109, 95, 79, 67, 53, 39.

Scheme 1. Microbial transformation result of 2,5-dimethyl-1,4-benzoquinone (DMBQ)



Anticandidal activity

The *in vitro* evaluation against the human pathogenic *Candida* sp. panel suggested that both the substrate and metabolite were mild in antifungal activity when compared to the standard azole in Table 1.

Table 1. Anticandidal activity (MIC, µg/mL)

Test Samples	*C. albicans* (clin)	*C. albicans* ATCC 24433	*C. glabrata* ATCC 2001	*C. glabrata* ATCC 66032	*C. tropicalis* ATCC 1369	*C. tropicalis* (clin)
Substrate DMBQ	16	8	16	8	16	4
Metabolite	32	16	32	16	32	8
Fluconazole	10	1.25	2.5	20	0.625	0.625

Antioxidant activity

According to *in vitro* results, the metabolite 2,5-dimethylhydroquinone showed 70.7% inhibition at 250 µg/mL concentration comperatively with gallic acid (positive control). The substrate (2,5-dimethyl-1,4-benzoquinone) at the same concentration was not effective on the DPPH scavenging activity.

Cytotoxicity testing

The metabolite was found to have an IC_{50} value of 6.71 µg/mL whereas IC_{50} value of substrate was calculated as <3.9 µg/mL against human skin fibroblast cell line.

As a conclusion, the natural bioactive derivative of 2,5-dimethyl-1,4-benzoquinone was obtained by using fungal biotransformation in 24 hours. This derivative was produced, to the best of our knowledge, for the first time by means of biotransformation in addition to its *in vitro* biological evaluation.

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